PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Linda H. MALKAS et al Confirmation No. **5889**

Appln. No.: **10/083,576** Group Art Unit: **0000**

Filed: **February 27, 2002** Examiner: **Unknown**

For: **METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING NUCLEAR ANTIGEN**

SUPPLEMENTAL DECLARATION OF DAVID L. MARKS

I the undersigned, David L. Marks, do hereby declare:

1. I, David L. Marks, am an Intellectual Property law Attorney, in TEC-COM, Office of Research and Development at the University of Maryland, Baltimore.

2. On January 9, 2003, I sent a letter to Dr. Pamela Bechtel, along with a copy of the above-captioned patent application (the specification, claims and drawings) and Declaration and Power of Attorney via FedEx Second Day Delivery, at her last known home address, 600 West Grove Parkway, Apt. 1110, Tempe, Arizona 85283-4550. The FedEx tracking number for this letter was 7912 7326 0212. A copy of this letter, and the FedEx Shipping Document therefor are attached hereto as Appendix A.

3. FedEx confirmed that on January 14, 2003, this letter was not delivered because the customer (Dr. Pamela Bechtel) was not available. A copy of the FedEx internet confirmation receipt for FedEx tracking number 7912 7326 0212 showing the same is attached hereto as Appendix B.

4. On January 22, 2003, this letter was returned to my office from FedEx. A copy of the FedEx internet confirmation receipt for FedEx tracking number 6057 5228 3419 showing the same is attached [illegible]

of Attorney for this application or because she can not be found or reached after diligent effort on my part to find or reach her.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

David L. Marks

January 22, 2002
Date

RESEARCH AND DEVELOPMENT

UNIVERSITY OF MARYLAND

January 9, 2003

VIA FED EX 2 Day

Pamela E. Bechtel, Ph.D.
600 West Grove Parkway, Apt. 1110
Tempe, AZ 85283-4550

Re: U.S. Patent Application 10/083,576
Title: Method for Purifying Cancer-Specific
Proliferating Cell Nuclear Antigen
Our Reference: LM-2000-051

Dear Dr. Bechtel:

Enclosed please find a copy of the above referenced patent application and a Declaration/Power of Attorney for that patent application. Please sign and return the Declaration/Power of Attorney to me as soon as possible. You can fax it to me at 410-706-1066.

Thank you for your time and assistance.

Sincerely;

David L. Marks

Enclosures
Malkas:LM2000-051:Patent:NP:CWPI-LM00-051-Declaration 6

RECEIVED

Docket No. A-7962

DECLARATION AND POWER OF ATTORNEY

As a below named co-inventor, We hereby declare that: Our residence, mailing address, and citizenship are as stated below next to our name. We believe we are an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD OF PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

the application of which

☐ is attached hereto OR ☑ was filed on February 27, 2002 as United States Application Number or PCT International Application Number 10/083,576 (Confirmation No. 5889), and was amended on ____________________ (if applicable).

We hereby state that we have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment specifically referred to above.

We acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part application(s), material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

We hereby claim foreign priority under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application(s) which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application(s) having a filing date before that of the application on which priority is claimed.

Prior Application Number(s)	Country	Filing Date	Priority Claimed Yes	Priority Claimed No
			☐	☑

We hereby claim benefit under 35 United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date

We hereby claim benefit under 35 United States Code §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in a listed prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112. We acknowledge my duty to disclose any information material to the patentability of this application as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. or International Application Number(s)	U.S. or International Filing Date	Status

We hereby appoint all attorneys of **SUGHRUE MION, PLLC** who are listed under the USPTO Customer Number shown below as my attorneys to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith, recognizing that the specific attorneys listed under that Customer Number may be changed from time to time at the sole discretion of Sughrue Mion, PLLC, and request that all correspondence about the application be addressed to the address filed under [illegible] USPTO C[illegible] Number.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and [illegible] further that these statements were made with the knowledge that willful false statements and the like [illegible]

NAME OF SOLE OR FIRST INVENTOR:

Given Name (first and middle [if any]) Linda H. | Family Name or Surname MALKAS

Inventor's Signature | Date

Residence: City Indianapolis | State IN | Country USA | Citizenship United States

Mailing Address: 6256 Lakeland Blvd.

City Indianapolis | State IN | Zip 46234 | Country United States

NAME OF SECOND INVENTOR:

Given Name (first and middle [if any]) Robert J. | Family Name or Surname HICKEY

Inventor's Signature | Date

Residence: City Indianapolis | State IN | Country USA | Citizenship United States

Mailing Address: 6256 Lakeland Blvd.

City Indianapolis | State IN | Zip 46234 | Country United States

NAME OF THIRD INVENTOR:

Given Name (first and middle [if any]) Pamela E. | Family Name or Surname BECHTEL

Inventor's Signature | Date

Residence: City Tempe | State AZ | Country USA | Citizenship United States

Mailing Address: 600 West Grove Parkway, Apt. 110

City Tempe | State AZ | Zip 85283 | Country United States

NAME OF FOURTH INVENTOR:

Given Name (first and middle [if any]) Min | Family Name or Surname PARK

Inventor's Signature Min Park | Date

Residence: City Los Alamos | State NM | Country USA | Citizenship Korean

Mailing Address: 116 San Juan Street

City Los Alamos | State NM | Zip 87544 | Country United States

NAME OF FIFTH INVENTOR:

Given Name (first and middle [if any]) Derek J. | Family Name or Surname HOELZ

Mailing Address [illegible] Knickerbocker Place, Apt. [illegible]

City Indianapolis | State IN | Zip [illegible]

NAME OF SOLE OR FIRST INVENTOR:

Given Name (first and middle [if any]) Linda H. | Family Name or Surname MALKAS

Inventor's Signature | Date

Residence: City Indianapolis | State IN | Country USA | Citizenship United States

Mailing Address: 6256 Lakeland Blvd.

City Indianapolis | State IN | Zip 46234 | Country United States

NAME OF SECOND INVENTOR:

Given Name (first and middle [if any]) Robert J | Family Name or Surname HICKEY

Inventor's Signature | Date

Residence: City Indianapolis | State IN | Country USA | Citizenship United States

Mailing Address: 6256 Lakeland Blvd.

City Indianapolis | State IN | Zip 46234 | Country United States

NAME OF THIRD INVENTOR:

Given Name (first and middle [if any]) Pamela E. | Family Name or Surname BECHTEL

Inventor's Signature | Date

Residence: City Tempe | State AZ | Country USA | Citizenship United States

Mailing Address: 600 West Grove Parkway, Apt. 110

City Tempe | State AZ | Zip 85283 | Country United States

NAME OF FOURTH INVENTOR:

Given Name (first and middle [if any]) Min | Family Name or Surname PARK

Inventor's Signature | Date

Residence: City Los Alamos | State NM | Country USA | Citizenship Korean

Mailing Address: 116 San Juan Street

City Los Alamos | State NM | Zip 87544 | Country United States

NAME OF FIFTH INVENTOR:

Given Name (first and middle [if any]) Derek J | Family Name or Surname HOLTZ

City In[illegible] | State IN | Zip [illegible]

NAME OF SOLE OR FIRST INVENTOR:

Given Name (first and middle [if any]) Linda H.	Family Name or Surname MALKAS
Inventor's Signature	Date

Residence: City Indianapolis	State IN	Country USA	Citizenship United States
Mailing Address: 6256 Lakeland Blvd.			
City Indianapolis	State IN	Zip 46234	Country United States

NAME OF SECOND INVENTOR:

Given Name (first and middle [if any]) Robert J.	Family Name or Surname HICKEY
Inventor's Signature	Date

Residence: City Indianapolis	State IN	Country USA	Citizenship United States
Mailing Address: 6256 Lakeland Blvd.			
City Indianapolis	State IN	Zip 46234	Country United States

NAME OF THIRD INVENTOR:

Given Name (first and middle [if any]) Pamela E.	Family Name or Surname BECHTEL
Inventor's Signature	Date

Residence: City Tempe	State AZ	Country USA	Citizenship United States
Mailing Address: 600 West Grove Parkway, Apt. 110			
City Tempe	State AZ	Zip 85283	Country United States

NAME OF FOURTH INVENTOR:

Given Name (first and middle [if any]) Min	Family Name or Surname PARK
Inventor's Signature	Date

Residence: City Los Alamos	State NM	Country USA	Citizenship Korean
Mailing Address: 116 San Juan Street			
City Los Alamos	State NM	Zip 87544	Country United States

NAME OF FIFTH INVENTOR:

Given Name (first and middle [if any]) Derek J.	Family Name or Surname HOELZ

Residence: City Indianapolis	State [illegible]	Country [illegible]	Citizenship [illegible]
Mailing Address: 3738 Knickerbocker Place, Apt. 3D			
[illegible]	[illegible]	[illegible]	[illegible]

NAME OF SIXTH INVENTOR:			
Given Name (first and middle [if any]) Dragana		Family Name or Surname TOMIC	
Inventor's Signature			Date
Residence: City Baltimore	State MD	Country USA	Citizenship United States
Mailing Address: 1 Fellowship Court, Apt. H			
City Baltimore	State MD	Zip 21286	Country United States

NAME OF SEVENTH INVENTOR:			
Given Name (first and middle [if any]) Lauren		Family Name or Surname SCHNAPER	
Inventor's Signature			Date
Residence: City Lutherville	State MD	Country USA	Citizenship United States
Mailing Address: 11801 Berans Road			
City Lutherville	State MD	Zip 21093	Country United States

METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

FIELD OF THE INVENTION

The present invention is directed to a method for purifying cancer-specific Proliferating Cell Nuclear Antigen (csPCNA), as well as to an ELISA for distinguishing csPCNA from native-proliferating cell nuclear antigen (nPCNA) and diagnosing cancer.

BACKGROUND OF THE INVENTION

A. Cancer

One of the least understood and most complex disease processes is the transformation that occurs as a cell becomes malignant. This process involves both genetic mutations and proteomic transformations, the result of which allows the cell to escape normal controls preventing inappropriate cell division. All cancers are unique and distinct from other cells, as well as other cancers. Despite this uniqueness, cancer cells share some common attributes. Most cancer cells proliferate outside of the normal cell cycle controls, exhibit morphological changes and exhibit various biochemical disruptions to cellular processes.

Cancer is usually diagnosed when a tumor becomes visible well after the first on-set of cellular changes. Many cancers are diagnosed after a biopsy sample is examined by histology for morphologic abnormalities, evidence of cell proliferation and genetic irregularities. There is

prognosis of malignancies. Many of the genetic and biochemical changes occur during the early development of a tumor and these changes should be exploited for the early diagnosis of cancer.

Breast cancer is the leading cause of death among women in the Western world. Recent data suggests that there is a strong correlation between late detection and poor prognosis of this disease. Analysis of a thousand clinical cases indicates that there is extensive genetic damage and a high rate of DNA synthesis in breast tumors in comparison with normal breast tissue. These data suggest that an alteration in the DNA replication machinery of breast cancer cells may contribute to uncontrolled and error-prone DNA synthesis.

Human breast cells mediate DNA synthesis using the multiprotein replication complex termed the DNA synthesome (Coll et al, *Oncology Research*, 8:435-447 (1996)). The DNA synthesome is fully competent to support *in vitro* DNA replication. The transformation of non-malignant human breast cells to a malignant state is accompanied by an alteration to a specific component of DNA synthesome, Proliferating Cell Nuclear Antigen (PCNA). PCNA is a well-known cell-cycle marker protein, originally identified as an antigen for autoimmune disease (Bechtel et al, *Cancer Research*, 58:3264-3269 (1998)).

B. PCNA in Cancer/Other Cell Processes

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Clinical Oncology, 121:122 (1995)). PCNA is a small (36 kD) nuclear protein involved in many cellular processes. PCNA plays crucial roles in both DNA replication and DNA repair mechanisms. PCNA has also been associated with transcription events. PCNA forms a trimer in the nucleus and acts as an accessory protein to polymerase δ, and also interacts with a variety of other proteins (Downey et al, *Cancer Cells*, 6:1211-1218 (1988)). In the evaluation of malignancy, PCNA is often used as a marker for cell proliferation. However, PCNA alone does not correlate with the stage of malignancy or the patient outcome.

A novel PCNA from breast cancer cells has been identified. The malignant breast cancer cells express a unique, acidic form of PCNA protein, i.e., csPCNA, which can clearly be distinguished from the basic form of this protein found in non-malignant cells, i.e., nPCNA. This alteration is most likely the result of a post-translational modification (Bechtel et al, *Cancer Res.*, 58:3264-3269 (1998)). However, prior to the present invention an effective method to purify csPCNA has not been described.

Recent advances in biochemical and genetic studies strongly indicate that PCNA may interact with different proteins involved in DNA mismatch repair, Okazaki fragments ligation, DNA methylation and chromatin assembly (Balajee et al, *Mutat. Res.*, 404:3-11 (1998); Ceccotti et al, *Curr Biol*: 6:1528-1531 (1996); Chen et al, [illegible] [illegible]; Eki et al, J. Biol.

Chem., 266:3087-3100 (1991); Eki et al, *J. Biol. Chem.*, 267:7284-7294 (1992); Greene et al, Hum Mol Genet: 8, 2263-2273 (1999); Gu et al, *Nucleic Acids Res.*, 26:1173-1178 (1998); Henderson et al, *Embo J.*, 13:1450-1459 (1994); Johnson et al, *J. Biol. Chem.*, 271:27987-27990 (1996); Kelman, *Oncogene*, 14:629-640 (1997); Kolodner et al, *Curr. Opin. Genet. Dev.*, 9:89-96 (1999); Krude, *Curr. Biol.*, 9:R394-R396 (1999); Lee et al, *J. Biol. Chem.*, 266:22707-22717 (1991); Levin et al, *Proc. Natl. Acad. Sci., USA*, 94:12863-12868 (1997); Levin et al, *Curr. Biol.*, 10:919-922 (2000); Martini et al, *J. Cell. Biol.*, 143:563-575 (1998); Merrill et al, *Genetics*, 148:611-624 (1998); Mimura et al, *Genes Cells*, 5:439-452 (2000); Miura, *J. Radiat. Res. (Tokyo)*, 40:1-12 (1999); Moggs et al, *Mol. Cell. Biol.*, 20:1206-1218 (2000); Nishikawa et al, *Jpn. J. Cancer. Res.*, 88:1137-1142 (1997); Otterlei et al, *Embo J.*, 18:3834-3844 (1999); Pan et al, *Proc. Natl. Acad. Sci., USA*, 90:6-10 (1993); Schweitzer et al, *Genetics*, 152:953-963 (1999); Shibahara et al, *Cell*, 96:575-85 (1999); Sinicrope et al, *Clin. Cancer. Res.*, 4:1251-1261 (1998); Tom et al, *J. Biol. Chem.*, 276:24817-24825 (2001); Tomkinson et al, *Mutat. Res.*, 407:1-9 (1998); Tsurimoto, *Front. Biosci.*, 4:D849-D858 (1999); Umar et al, *Cell*, 87:65-73 (1996); and Wu et al, *Nucleic Acids Res.*, 24:2036-2043 (1996)).

Xeroderma Pigmentosum (XP)G protein is reported to interact with PCNA (Gary et al, *J. Biol. Chem.*,

PCNA-binding regions of FEN-1 and cyclin-dependent kinase inhibitor p21. XPG is a repair endonuclease similar to FEN-1 and required for nucleotide excision repair. The human XPG endonuclease cuts on the 3' since of a DNA lesion, during nucleotide excision repair.

In the present invention, XPG protein was unexpectedly found to be useful in selectively purifying csPCNA, and as a part of an ELISA system which can distinguish the csPCNA from the nPCNA. The detection of csPCNA in the ELISA serves as a powerful marker for early detection of malignancy.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for selectively purifying csPCNA.

Another object of the present invention is to provide an ELISA for detection of csPCNA and early detection of malignancy.

The above-described objects, as well as others, which will be apparent from the detailed description of the invention provided hereinafter, have been met in one embodiment by a method for purifying csPCNA comprising the steps of:

(A) obtaining a tissue or body fluid sample comprising csPCNA;

(B) contacting said sample with a peptide comprising the amino acid sequence

LeuLysGlnLeuAspAlaGlnGlnThrGlnLeuArg

wherein said peptide is immobilized

on a solid support and binds to said csPCNA to form a peptide-csPCNA complex; and

(C) isolating csPCNA from said peptide-csPCNA complex so as to purify said csCNA.

In another embodiment, the above-identified objects have been met by an immunoassay for detecting csPCNA comprising:

(1) contacting a test sample with a peptide comprising the amino acid sequence LeuLysGlnLeuAspAlaGlnGlnThrGlnLeuArgIle AspSerPhePheArgLeuAlaGlnGlnGluLysGluAsp AlaLysArg (SEQ ID NO:1), which has been immobilized on a solid support so as to bind csPCNA to said peptide to form a peptide-csPCNA complex; and

(2) contacting said peptide-csPCNA complex with an anti-PCNA antibody and detecting binding of said antibody to said complex.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a flow chart depicting steps involved in the isolation of total PCNA and csPCNA.

Figure 2 shows a Western blot of fractions collected during the isolation of PCNA. The fractions were separated on a 10% (w/v) SDS-PAGE gel, transferred to nitrocellose and analyzed using anti-PCNA antibodies. Lane 1: H. Lane 2: S1.

Lane 9: PSW; Lane 10: PSE; Lane 11: QS; Lane 12: XPGS; Lane 13: XPGE.

Figure 3 shows a Coomasie stained gel of fractions collected during the isolation of PCNA. The fractions were separated on a 10% (w/v) SDS-PAGE gel, and then stained with Coomasie blue. Lane 1: H; Lane 2: S1; Lane 3: S2; Lane 4: NE/S3; Lane 5: PCFT; Lane 6: PCLS; Lane 7: PCHS; Lane 8: PSFT; Lane 9: PSW; Lane 10: PSE; Lane 11: QS; Lane 12: XPGS; Lane 13: XPGE.

Figure 4 shows the results of XPGE which was subjected to 2-dimensional SDS-PAGE and Western blot analysis to identify which form of PCNA was present.

Figure 5A shows the results of a validation study on the viability of the streptavidin surface of streptavidin-coated plates.

Figure 5B shows the results of a study for determining the maximal binding capability of biotinylated XPG-GST protein to streptavidin-coated plates.

Figure 6 shows denisitometric analyses of total PCNA in P4 fractions from MCF7 and MCF10A cells.

Figure 7A-7C show the results of ELISAs using MCF7 P4 and MCF10A P4 proteins.

Figure 8 shows the results of an ELISA assay comparing the abundance of csPCNA in extracts of MCF7 (malignant) cells and MCF 10A (normal) cells.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, in one embodim

have been met by a method for purifying csPCNA comprising the steps of:

(A) obtaining a tissue or body fluid sample comprising csPCNA;

(B) contacting said sample with a peptide comprising the amino acid sequence LeuLysGlnLeuAspAlaGlnGlnThrGlnLeuArg IleAspSerPhePheArgLeuAlaGlnGlnGluLys GluAspAlaLysArg (SEQ ID NO:1), wherein said peptide is immobilized on a solid support and binds to said csPCNA to form a peptide-csPCNA complex; and

(C) isolating csPCNA from said peptide-csPCNA complex so as to purify said csCNA.

Preferably, prior to step (B), the thus obtained tissue or body fluid sample of step (A) is subjected to a process comprising the steps of:

(1) homogenizing cells constituting said tissue or body fluid to obtain a homogenate (H);

(2) separating said H into a nuclear pellet fraction (NP) and a cytosolic fraction (S1);

(3) extracting nuclei from said NP to obtain a nuclear extract (NE);

(4) subjecting said S1 to centrifugation to obtain a

(5) subjecting said S2 to centrifugation to obtain a post-mitochondrial/post-microsomal cytosolic supernatant (S3);

(6) combining said NE and said S3 to form an NE/S3 fraction, applying the resulting NE/S3 fraction to a phosphocellulose (a weak anion exchange matrix) column and collecting the flow through (PCFT);

(7) applying the resulting PCFT to a phenylsepharose (a hydrophobic chromatography matrix) column, eluting the column with buffer comprising ethylene glycol and collecting the eluant (PSE);

(8) dialyzing out ethylene glycol present in the PSE to obtain a dialyzate; and

(9) applying the resulting dialyzate to a Q-Sepharose (a strong anion exchange matrix) column, eluting with a dialyzate buffer comprising a salt gradient, and collecting and pooling PCNA-containing fractions to obtain said sample.

More preferably, the tissue or body fluid sample of step (A) further comprises native-PCNA (nPCNA), said nPCNA does not bind to said peptide in step (B) but flows through the column (XPGS), whereas csPCNA binds to said peptide in step (B) to form a peptide-csPCNA complex and [illegible]

The particular tissue or body fluid sample which is employed in the present invention is not critical thereto. Examples of tissue which can be employed in the present invention include cervical, mammary glands, esophageal, glial cells, lung, stomach, intestine, prostate, and white blood cells. Examples of body fluid which can be employed in the present invention include urine, serum and whole blood.

The source of the tissue or body fluid is from a subject afflicted with a cancer. The particular cancer is not critical to the present invention. The cancers can be carcinomas, sarcomas, lymphomas, or leukemias. Examples of such cancers include cervical carcinoma, mammary gland carcinoma of ductal or lobular origin, gliomas, prostate, lung, esophageal, stomach, and ovarian cancer.

In step (B) the solid support employed is critical to the present invention, because the XPG peptide of SEQ ID NO:1 can be expressed as a fusion protein, e.g., a GST fusion (GST = glutathione-S-transferase), and the support will depend on the fusion partner. An example of the solid support which can be employed in this case include Glutathione Sepharose (Pharmacia). However, expression of the XPG-fusion protein in a Calmodulin or 6x His (oligo (6X) histidine) format can also be used in the present invention.

In step (C), csPCNA is isolated from the complex by, for example, [illegible]

[illegible] through the column and appears in the flow-through

liquid exiting the column while the complex and contaminating proteins are being loaded onto the column.

The peptide represented by SEQ ID NO:1 may be synthesized chemically or by recombinant DNA techniques, as described by Gary et al, *J. Biol. Chem.*, 232:24522-24529 (1997). Further, as noted above, the peptide may be in the form of a fusion protein. The partner of the fusion protein is not critical to the present invention. Examples of such partners include Glutathione-S-Transferase (GST), Calmodulin Binding protein, and oligo(6X) histidine.

The resulting preferred csPCNA can be used to produce antibodies (monoclonal or polyclonal) specific for csPCNA by conventional techniques. The resulting purified csPCNA can also be used as standards for diagnostic kits, as well as enabling development of specific inhibitors for csPCNA (not nPCNA), the identification of the site(s) on the PCNA polypeptide that is (are) modified in nPCNA, provide the baseline for comparison to identify the type of modification sustained by nPCNA and lacking from csPCNA, the identification of specific metabolic pathways that mediate the addition or removal of this (these) post-translational modifications.

Products of antibodies specific for csPCNA can be produced by challenging mammals (e.g., rabbits, goats, horses, etc) with the peptide sequence which is post-translationally [illegible]

interdomain connector loop. They cannot distinguish

csPCNA from nPCNA. Identification of the amino acid sequence of PCNA that is bound by the XPG peptide only in the csPCNA form of PCNA provides the target for preparing selective antibodies recognizing only csPCNA.

Specific inhibitors of csPCNA's interaction with their target proteins can be produced by construction or expression of peptides identical to the interacting domains of csPCNA and by computational chemistry methods. Through computational chemistry, the sites of interactions can be modeled and searches of existing 3-D chemical library structures or the design of new compounds can be made to disrupt and/or promote interaction between csPCNA and the proteins.

Using mass spectrometry peptide analysis of tryptic fragments of csPCNA and nPCNA will identify the fragments (peptides) that are unique to csPCNA. Sequence identification of these peptides using LC/MS-TDF mass spectrometry will identify the sequence, and thus the position of the modified peptides or amino acids within nPCNA that are modified in csPCNA (or that are modified in csPCNA and are not modified in nPCNA).

Furthermore, molecular weight and sequence analysis of these peptides by mass spectroscopy will indicate what types of post-translational modifications have been sustained by csPCNA and/or nPCNA. The identification of these post-translational modifi[illegible]

[illegible] the loss of the modification from

the csPCNA. In addition, computer analysis of the amino acid domains of the csPCNA that interact with the XPG peptide can reveal the presence of specific consensus sequences for a particular type of post-translational modification.

csPCNA is believed to be a better diagnostic/prognostic indicator for cancer than current methodologies which measure total PCNA. This is because total PCNA of malignant tissue/cells takes into account nPCNA and csPCNA, while this method of diagnostic/prognostic indication detects a form of PCNA only found in malignant cells/tissue. Based upon the abundance of the cancer specific form of csPCNA relative to the abundance of nPCNA the degree or extent of malignancy can be estimated. Using an ELISA assay, e.g., one whose results are shown in Figure 8, the abundance of PCNA can be measured relative to a set of standards of csPCNA of varying abundance. Comparison of the experimentally determined absorbance (using the ELISA) for the sample to the absorbance of the standards can be used to indicate the abundance of the csPCNA in the sample.

Accordingly, in another embodiment, the above-described objects of the present invention have been met by an immunoassay for detecting csPCNA comprising:

(1) contacting a test sample with a peptide comprising the amino acid sequence [illegible]

immobilized on a solid support so as to

bind csPCNA to said peptide to form a peptide-csPCNA complex; and

(2) contacting said peptide-csPCNA complex with an anti-PCNA antibody and detecting binding of said antibody to said complex.

The particular test sample employed is not critical to the present invention and may include any of the tissue or body fluid samples discussed above.

The particular format of the immunoassay of the present invention is not critical to the present invention. Examples of such formats include an ELISA, radio-immuno assay, dot blot assay, slot blot assay, immunoprecipitation and protein quantification, immuno-PCR, and Western blot.

Antibodies to PCNA can be prepared by challenging mammals (e.g., rabbits, goats, horses, etc) with the peptide sequence which is post-translationally modified in nPCNA, but not in csPCNA. All commercially available antibodies to PCNA that exist to date (~10) recognize the interdomain connector loop. They cannot distinguish csPCNA from nPCNA. Identification of the amino acid sequence of PCNA that is bound by the XPG peptide only in the csPCNA form of PCNA provides the target for preparing selective antibodies recognizing only csPCNA.

The detectable enzyme employed in the ELISA is

As discussed above, the partner of the fusion protein is not critical to the present invention and examples of such partners include GST, Calmodulin Binding Protein and oligo(6X)histadine.

The particular mode of immobilization of the fusion protein on the solid support is not critical to the present invention.

It is preferable that the fusion protein is immobilized on the solid support via biotin-streptavidin conjugation.

The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

EXAMPLE 1
Isolation of Total PCNA

Total PCNA was isolated using a series of centrifugation and chromatographic steps as shown in Figure 1, and discussed in detail below.

A. Nuclear Extract (NE)

Human breast cancer cells, MCF7 cells (ATCC No. HTB-22), were grown in DMEM which was supplemented with 10% (v/v) fetal bovine serum, 1.0% (w/w) penicillin/streptomycin, and non-essential amino acids, and then frozen until use.

13.9 g of MCF7 cells were resuspended in 1 volume of homogenization buffer comprising [illegible]

[illegible] strokes. The homogenate H was then centrifuged at 3000 rpm for 10 min, the cytosolic supernatant

removed (S1) and 10 ml of nuclear extraction buffer comprising 50 mM KCl, 50 mM HEPES (pH 7.5), 5.0 mM $MgCl_2$, 5.0 mM EDTA, 5.0 mM EGTA, 1.0 mM DTT and 0.1 mM PMSF, was added to the nuclear pellet.

The resulting nuclear pellet was rocked at 4°C for 2 hour. The nuclear pellet was centrifuged at 2,500 rpm for 10 min. The resulting supernatant was removed and centrifuged at 100,000 x g in a Ti50.2 rotor for 1 hr. The resulting supernatant, i.e., nuclear extract (NE), was collected.

EDTA and EGTA were added to S1 to 5.0 mM, and the resulting fraction was centrifuged in an SS34 rotor at 17,000 x g for 15 min. The resulting post-mitochondrial supernatant (S2) was collected, and centrifuged at 100,000 x g for 1 hr in a Ti50.2 rotor. The resulting post-mitochondrial and post-microsomal cytosolic supernatant (S3), was collected.

The NE was then combined with the S3 to obtain an NE/S3 fraction.

B. Phosphocellulose Column

Phosphocellulose (PC) was resuspended in a low salt buffer (LS) comprising 200 mM KCl, 50 mM HEPES (pH 7.5), 5.0 mM $MgCl_2$, 1.0 mM DTT and 0.1 mM PMSF. A 20 ml column was poured and attached to a BioRad Biologic system. 25 ml of the NE/S3 fraction was loaded onto the column, and the flow through (PCFT) collected. A low salt fraction (PCLS) was then

[illegible]

salt buffer (HS) comprising 1.0 M KCl, 50 mM HEPES

(pH 7.5), 5.0 mM $MgCl_2$, 1.0 mM DTT and 0.1 mM PMSF. The fractions were analyzed by Western blot analysis as described below, to determine which fractions contained PCNA.

C. Phenylsepharose Column

A phenylsepharose column (PS) was prepared as directed by the manufacturer (Sigma Chemical Co.) and a 6.0 ml column was poured. The column was incubated with 40 ml pre-equilibration buffer comprising 20 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.1 mM EGTA, 10% (v/v) glycerol, 1.0 M ammonium sulfate and 1.0 mM DTT, then the identified PCNA-containing fraction from the PC column was adjusted to 1.0 M ammonium sulfate and applied to the PS column. The PS column was washed with 40 ml of wash buffer comprising 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, 10% (v/v) glycerol, 0.5 M ammonium sulfate and 1.0 mM DTT (PSW), and the flow through collected (PSFT). Fractions were eluted with 40 ml of elution buffer comprising 20 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 0.1 mM EGTA, 20% (v/v) glycerol, 10% (v/v) ethylene glycol and 1.0 mM DTT (PSE).

The PSE fractions were dialyzed in a 0.5 M KCl buffer comprising 0.5 M KCl, 50 mM HEPES (pH 7.5), 1.0 mM of protease inhibitor cocktail (Sigma Chemical and/or Behringer-Mannheim and/or Calbio-Chem), 5.0 mM $MgCl_2$ and 1.0 mM DTT; a 0.2 M KCl buffer

Calbio-Chem , 5.0 mM $MgCl_2$ and 1.0 mM DTT; and TDEG

buffer comprising 50 mM Tris (pH 7.5), 1.0 mM DTT, 1.0 mM EDTA, and 10% (v/v) glycerol, and containing 100 mM KCl. The fractions were then analyzed by Western Blot, as described below, to determine which fractions contained PCNA, and the PCNA-containing fractions were combined.

D. Q-Sepharose Column

A 5.0 ml Q-Sepharose column (BioRad) was attached to a BioRad Biologic system. The combined PCNA-containing fractions from the PS column were concentrated to 12.5 ml, and then applied to the column. Fractions (1.0 ml) were eluted using TDEG buffer with a salt gradient of 0.1 M KCl to 0.6 M KCl. The resulting fractions (QS) were analyzed by Western blot, as described below, and the PCNA-containing fractions were combined.

E. Western Blot Analysis

Western blot analysis was performed as described by Bechtel et al, *Cancer Res.*, 58:3264-3269 (1998). Specifically, Western blot analysis was performed using an antibody against PCNA (Amersham) 1:1000, anti-mouse 1:3000 (Amersham) and detection using chemiluminesence (Pharmacia).

The fractions collected at all of the steps of the purifications process were run on a 10% (w/v) SDS-PAGE gel, transferred to nitrocellulose and examined by Western blot analysis (Figure 2) using an antibody [illegible]

of the fractions through the phosphocellulose flow

through (PCFT). Low levels of PCNA were found in phosphocellulose low salt (PCLS) and high salt (PCHS) fractions, but were undetectable in the 10 μg/ml samples loaded onto the gel. PCNA becomes concentrated in the phenylsepharose eluant (PSE) and Q-sepharose (QS) fractions. The stained gels (Figure 3) illustrate that at each step, PCNA was further purified through the Q-sepharose column.

EXAMPLE 2
Isolation of csPCNA

The purified total PCNA from the Q-sepharose column was subjected to extraction using a 29 amino acid peptide (SEQ ID NO:1) derived from protein XPG, which had been fused to a GST tag. This portion of the XPG protein, i.e., amino acids 981-1009, has been shown to bind PCNA (Gary et al, *supra*).

A. Isolation of GST-XPG Fusion Protein

A peptide fragment of the protein XPG was cloned into *E. coli* as a GST fusion protein, as described by Gary et al, *supra*.

The transformed bacteria were grown overnight in a shaker at 37°C in 500 ml of Terrific broth comprising 23.5 g Terrific broth powder (Life Technologies Inc., Gaithersburg, MD), 2.0 ml glycerol and 100 μg/ml Ampicillin. GST expression was induced by adding 0.1 IPTG to the culture and incubating for 4 hrs. The bacteria were collected by centrifugation in a GS lite rotor at [illegible]

The pellet was resuspended in B-Per (Pierce, Rockford, IL), rocked at room temperature for 10 min

and centrifuged at 27,000 x g for 10 min in a SS34 rotor. The supernatant, i.e., bacterial lysate, was collected.

Glutathione Sepharose 4B matrix beads were resuspended in the storage buffer supplied by the manufacturer, 2.0 ml of the resuspended beads were washed 10 volumes of 4°C PBS, pelleted, and then the beads were resuspended in 1.0 ml of cold PBS. The resulting GST beads were incubated with the bacterial lysate for 30 min at room temperature and centrifuged in a table top centrifuge at 2500 rpm to obtain GST-XPG-glutathione beads.

B. Purification of PCNA

The resulting GST-XPG-glutathione beads were equilibriated in $T_{50}K_{300}/P_{100}$ buffer comprising 50 mM Tris (pH 7.5), 300 mM KCl and 100 mM potassium phosphate (pH 7.4). The beads were then incubated with the PCNA-containing fractions from the Q-Sepharose column for 30 min at 4°C and centrifuged at 2500 rpm in a table top centrifuge. The supernatant was decanted and the beads were washed with equilibriation buffer comprising 50 mM Tris (pH 7.5), 300 mM KCl and 100 mM potassium phosphate (pH 7.4) (XPGS). PCNA was eluted by incubating the beads with elution buffer comprising 50 mM Tris (pH 7.5) for 30 min at 4°C, centrifuging in a microfuge and collecting the supernatant (XPGE). The wash (XPGS) and eluant (XPGE) were examined by

As shown in Figure 2, both XPGE and XPGS fractions contained PCNA. Further, as shown in Figure 3, only a single protein was present in the XPGE fraction and only a few proteins were present in the XPGS fractions.

C. <u>2D PAGE</u>

The XPGE was further analyzed by 2D PAGE as described below, to determine which form of PCNA was present.

2D PAGE was performed as described by Bechtel et al, *supra*. Specifically, XPGE (20-40 μg of protein) was loaded onto a first-dimension tube gel comprising 9.2 M urea, 4.0% (w/v) acrylamide, 2.0% (v/v) ampholytes (pH 3-10), and 20% (v/v) Triton X-100. The polypeptides were separated along a pH gradient created using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were then placed onto an 8.0% (w/v) acrylamide-SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then examined by Western blot as described above. The results are shown in Figure 4.

As shown in Figure 4, csPNCA is the only form of PCNA present in the XPGE fraction.

D. <u>Polymerase Assays</u>

DNA polymerase δ activity was measured as described by Han et al, *Biochem. Pharm.*, <u>60</u>:403-411 (2000). csPCNA has the ability to stimulate the

[illegible]

template, at a concentration of 0.2 OD_{260} units/ml,

and the reaction mixture contained 1.0-2.0 μg of purified DNA synthesome protein, 10 mM $MgCl_2$, 10 mM dCTP, 25 mM HEPES (pH 5.9), 200 $\mu g/ml$ of bovine serum albumin, 100 $\mu Ci/ml$ of [^{32}P]dGTP, and 5.0% (v/v) glycerol. The poly[dG-dC]/[dG-dC] template was boiled for 5 minutes and chilled on ice prior to use in the assay. The reaction mixture containing these components was incubated at 37°C for 15 minutes, and then spotted onto Whatman DE81 filters (Whatman International Ltd, Maidstone, England). The amount of radiolabelled nucleotide bound to the filters was quantified after washing the filters with 10 ml per filter of 0.3 M NaPPi (pH 7.5); followed by washing the filters three times with 10 ml per filter of 0.1 M NH_4 formate (pH 7.4). Afterwards, the filters were given a final wash in 95% (v/v) ethanol and then air dried, placed in scintillation vials, covered with 3 ml of scintillation fluid, and placed in a Packard TriCarb 2100TR scintillation counter (Packard Instruments Co., Meriden, CT).

The results of this assay demonstrate that addition of PCNA to purified polymerase δ increases the processivity of polymerase δ (See Bravo et al, *Nature*, <u>326</u>:515-517 (1987); Downey et al, *Cancer Cells*, <u>6</u>:1211-1218 (1988); and Tan et al, *Proc. Natl. Acad. Sci., USA*, <u>90</u>:11014 (1986)). The addition of csPCNA to purified polymerase δ was found to increase its processitivity as reported

[illegible]
contains bound PCNA.

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EXAMPLE 3

ELISA Assay

A. Isolation of GST-XPG Fusion Protein

The PCNA binding domain of XPG (SEQ ID NO:1) was ligated into a pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Recombinant protein was expressed in BL21 (DE3) *Escherichia coli* using 0.8 mM IPTG. Cells were lysed using B-Per reagents (Pierce, Rockford, IL); the lysate was incubated with glutathione-agarose beads for 2 hours at 4°C and subsequently centrifuged for 10 minutes at 3000 rpm. The lysate was washed twice with PBS followed by elution with 10 mM reduced glutathione (Sigma, Co., St. Louis, MO) in 50 mM Tris-HCl (pH 7.4).

The resulting purified XPG-GST was biotinylated using a commercial ECL protein biotinylation kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ). Briefly, 1.0 mg of protein was diluted in 1.0 ml of biocarbonate buffer (pH 8.6), and incubated for 1 hour at room temperature, in 30 μl of biotinylation reagent per mg of protein. After incubation, the protein sample was applied to a Sephadex G25 column and eluted with 5.0 ml of PBS (pH 7.4). Fractions of biotinylated XPG-GST protein were then collected.

The protein profile of the biotinylated XPG-GST protein was analyzed by 12% (w/v) SDS-PAGE and Silver-Stain procedure (Bio-Rad silver stained plus kit, Bio-Rad, Hercules, CA). To determine the

was used at a [illegible] dilution, followed by HRP-labeled anti-goat IgG (Santa Cruz Biotechnology,

Inc., Santa Cruz, CA) at a 1:12,000 dilution in 10% (v/v) blocking buffer. The presence of biotinylated fraction of XPG-GST protein was detected using streptavidin-horse radish peroxidase conjugated protein (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) at dilution of 1:6,000 in PBST. Immunodetection was performed using ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The biotinylated XPG-GST was seen as a 32 kDa discrete protein band.

To determine a suitable concentration of biotinylated XPG-GST protein which may be enough to saturate all streptavidin-binding sites on wells of a 96-well streptavidin-coated plate (Pierce, Rockford, IL), a validation of the streptavidin surface of streptavidin-coated plate was first carried out using biotinylated-HRP enzyme. Specifically, different dilutions of biotinylated enzyme HRP (biotin-HRP) (Sigma, Co., St. Louis, MO) stock solution were incubated for 1 hour at room temperature, washed 5 times with PBS (pH 7.4) containing 0.1% (v/v) Tween20). Then, 100 μl of substrate solution for HRP enzyme (Sigma Co., St. Louis, MO) was added, followed by 15 minutes of incubation at 25°C. To stop the reaction, 50 μl of stop solution (0.5 M H_2SO_4) was added. Absorbance was measured at 450 nm. Streptavidin-HRP (Sigma Co., St. Louis, MO) was used at different dilutions in 0.1% (v/v) polyoxyethylenesorbitoan Monolaurate

As shown in Figure 5A, streptavidin-coated plates were found to be completely saturated by biotin-HRP even at 1:150,000 dilution per well.

Since the streptavidin-coated plate was completely saturated by biotin-HRP at 1:150,000 dilution, the same dilution of this enzyme was used for determination of a suitable concentration of biotinylated XPG-GST protein to use. Specifically, streptavidin-coated plate was first incubated for 1 hour with different concentrations of biotinylated XPG-GST protein (10-150 μg/ml) at 25°C. After washing 5 times with PBST, biotin-HRP was added in a 1:150,000 dilution. HRP-linked enzyme was detected by adding 3,3',5,5'-tetramethylbenzidin liquid substrate system for ELISA (Sigma) of substrate-reacting solution, and absorbance was measured at 450 nm. The results are shown in Figure 5B.

As shown in Figure 5B, the optimal concentration of biotinylated XPG-GST protein that was required for saturation of all of the streptavidin-binding sites on the plate was 100 μg/ml.

B. <u>ELISA</u>

A streptavidin-coated plate was incubated with 100 μg/ml of biotinylated XPG-GST protein for 1 hour at 25°C. Then, the wells of the plate were washed with PBST and incubated with different dilutions of protein [illegible]

MI) breast cell lines. The protein samples were obtained as follows.

MCF7 and MCF10A were cultured according to the protocols provided by the American Type Culture Collection (ATCC). Briefly, MCF7 cell cultures were maintained in Dulbecco's Modified Essential Media (DMEM) supplemented with 5.0% (v/v) fetal bovine serum (FBS), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 1.0% (w/v) non-essential amino acids. MCF10A cultures were maintained in DMEM/F12 supplemented with 5.0% (v/v) Ca^{++} horse serum, 10 mM HEPES, 10 μg/ml of insulin, 20 ng/ml of epidermal growth factor (EGF), 100 ng/ml of cholera enterotoxin, 0.5 μg/ml of hydrocortisone, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Both cell types were grown as monolayers at 37°C, in 5.0% CO_2 atmosphere. Semiconfluent (50-75%) cell cultures were harvested and washed three times with phosphate-buffered saline (PBS), and then pelleted using low-speed centrifugation, i.e., 200 x g for 5 min at 4°C. The cell pellets were stored at -80°C until use. Next, the DNA synthesome-enriched fraction, i.e., the P4 faction, was isolated from the pellets of the non-malignant (MCF10A) and malignant (MCF7) breast cell lines as described by Coll et al, *Oncol. Res.*, 8(10,11):435-447 (1996), and the protein concentration in the P4 fraction was determined by a colorimetric assay.

densitometric analysis. More specifically, 5-300 μg

of the samples were resolved in 12% (w/v) SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using a monoclonal PCNA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The values after scanning Western blot fluorograms, were expressed in Arbitary units (Au). Discrete protein bands were quantified using BIORAD GS 710 Imaging Densitometer. The results of the densitometric analyses are shown in Figure 6, which reflects the average of three independent assays, expressed as means ± SE.

As shown in Figure 6, the results demonstrate that the P4 fraction isolated from MCF7 cells contains twice the PCNA protein than that found in the P4 fraction isolated from MCF10A cells. Even when this result was taken in consideration as discussed below, XPG peptide was still able to distinguish csPCNA in the two cell lines.

Thereafter, dilutions of the P4 fraction from MCF7 and MCF10A cells were added to the biotinylated XPG-GST coated plates. Incubation was performed overnight at 4°C in buffer comprising 20 mM Tris-HCl (pH 7.4), 60 mM NaCl, 300 mM KCl, and 100 mM KPO_4 as described by Gary et al, supra. After washing with PBST, the wells were incubated with PCNA antibody labeled with HRP enzyme, for 1 hour at room temperature with constant agitation. The HRP-conjugated monoclonal PCNA antibody was obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and was used [illegible]

detected with TMB (3,3',5,5' tetramethylbenzidin)

(Sigma) substrate and absorbency was read at 450 nm. The results, which are shown in Figure 7A, reflect the average of three independent assays, expressed as means ± SE.

As shown in Figure 7A, XPG peptide is capable of distinguishing two forms of PCNA in the ELISA assay.

Next, serial dilutions of the P4 proteins were tested in duplicate in the ELISA and the mean value of absorbency was calculated and used for comparisons. Standard curves, representing the correlation between absorbency and the abundance of the malignant and non-malignant form of PCNA, were prepared and compared to each other. The results, which are shown in Figure 7B (MCF7) and Figure 7C (MCF10A), represented the mean values from three independent experiments.

As shown in Figures 7B and 7C, the XPG peptide was found to have a higher binding affinity for csPCNA present in MCF7 breast cell lines.

Next, MCF10A and MCF7 cells were grown as described above. Semi-confluent (50-75%) cell cultures were harvested and washed three times with PBS, and then pelleted using low-speed centrifugation, i.e., 200 x g for 5 min at 4°C. The cell pellets were stored at -80°C until use. Next, the DNA synthesome-enriched fraction, i.e., the P4 faction, was isolated from the pellets of the non-malignant (MCF10A) and malignant (MCF7) breast cell lines [illegible]

[illegible]

cells was analyzed by ELISA. Specifically, the

P4 fraction was added to biotinylated XPG-GST coated plates. Incubation was performed overnight at 4°C in the buffer comprising 20 mM Tris-HCl (pH 7.4), 60 mM NaCl, 300 mM KCl, 100 mM KPO_4 as described above. After washing with PBST, the wells were incubated with PCNA antibody (Santa Cruz Biotechnology, Inc.), labeled with HRP enzyme, for 1 hour at room temperature with constant agitation. The PCNA antibody was used at dilution of 1:500 in 10% (v/v) blocking buffer comprising PBS (pH 7.4), 0.1% (w/v) BSA and 0.05% (v/v) Tween20. HRP enzyme was detected with TMB (3,3',5,5'-tetramethylbenzidin) (Sigma) substrate and absorbency was read at 450 nm. The amount of PCNA is indicated in arbitrary units. The results are shown in Figure 8.

As shown in Figure 8, and similar to the results in Figure 6, the P4 fraction isolated from MCF7 cells contains twice the PCNA protein than that found in the P4 fraction isolated from the MCF10A cells. Even when this result was taken in consideration (where 10 μg of the P4 fraction isolated from MCF10 cells = 1 arbitrary unit PCNA; and 5.0 μg of the P4 fraction isolated from MCF7 cells = 1 arbitrary unit PCNA), XPG peptide was still able to distinguish csPCNA in the two cell lines.

While the invention has been described in detail and with reference to specific embodiments [illegible]

[illegible] thereof.

WHAT IS CLAIMED:

Claim 1. A method for purifying cancer-specific (csPCNA) comprising the steps of:

(A) obtaining a tissue or body fluid sample comprising csPCNA;

(B) contacting said sample with a peptide comprising the amino acid sequence LeuLysGlnLeuAspAlaGlnGlnThrGlnLeuArg IleAspSerPhePheArgLeuAlaGlnGlnGluLys GluAspAlaLysArg (SEQ ID NO:1), wherein said peptide is immobilized on a solid support and binds to said csPCNA to form a peptide-csPCNA complex; and

(C) isolating csPCNA from said peptide-csPCNA complex so as to purify said csCNA.

Claim 2. The method of Claim 1, wherein prior to step (B) the thus obtained tissue or body fluid sample of step (A) is subjected to a process comprising:

(1) homogenizing cells constituting said tissue or body fluid to obtain a homogenate (H);

(2) separating said H into a nuclear pellet fraction (NP) and a cytosolic fraction (S1);

(3) extracting nuclei from said NP to obtain a nuclear extract (NE

post-mitochondrial cytosolic supernatant (S2);

(5) subjecting said S2 to centrifugation to obtain a post-mitochondrial/post-microsomal cytosolic supernatant (S3);

(6) combining said NE and said S3 to form an NE/S3 fraction, applying the resulting NE/S3 fraction to a weak anion exchange matrix column and collecting the flow through (PCFT);

(7) applying the resulting PCFT to a hydrophobic chromatography matrix column, eluting the column with buffer comprising ethylene glycol and collecting the eluant (PSE);

(8) dialyzing out ethylene glycol present in the PSE to obtain a dialyzate; and

(9) applying the resulting dialyzate to a strong anion exchange matrix column, eluting with a dialyzate buffer comprising a salt gradient, and collecting and pooling PCNA-containing fractions to obtain said sample.

Claim 3. The method of Claim 1 or 2, wherein said tissue or body fluid sample of step (A) further comprises native PCNA (nPCNA), said nPCNA does not bind to said peptide in step (B), whereas said

csPCNA is eluted from said csPNA-complex.

Claim 4. An immunoassay for detecting csPCNA comprising:

(1) contacting a test sample with a peptide comprising the amino acid sequence LeuLysGlnLeuAspAlaGlnGlnThrGlnLeuArgIle AspSerPhePheArgLeuAlaGlnGlnGluLysGluAsp AlaLysArg (SEQ ID NO:1), which has been immobilized on a solid support so as to bind csPCNA to said peptide to form a peptide-csPCNA complex; and

(2) contacting said peptide csPCNA complex with an anti-PCNA antibody and detecting binding of said antibody to said complex.

Claim 5. The immunoassay of Claim 4, wherein said assay is an ELISA and said antibody is labeled with a detectable enzyme.

Claim 6. The immunoassay of Claim 5, wherein said enzyme is horse radish peroxidase.

Claim 7. The immunoassay of Claim 5, wherein said peptide is a fusion protein comprising said peptide and Glutathione-S-Transferase.

Claim 8. The immunoassay of Claim 7, wherein said fusion protein is biotinylated and immunobilization on said solid support via streptavidin-coated on said solid support.

METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

ABSTRACT

A method for purifying cancer-specific Proliferating Cell Nuclear Antigen (csPCNA) is described, as well as an immunoassay based thereon.

FIGURE 1

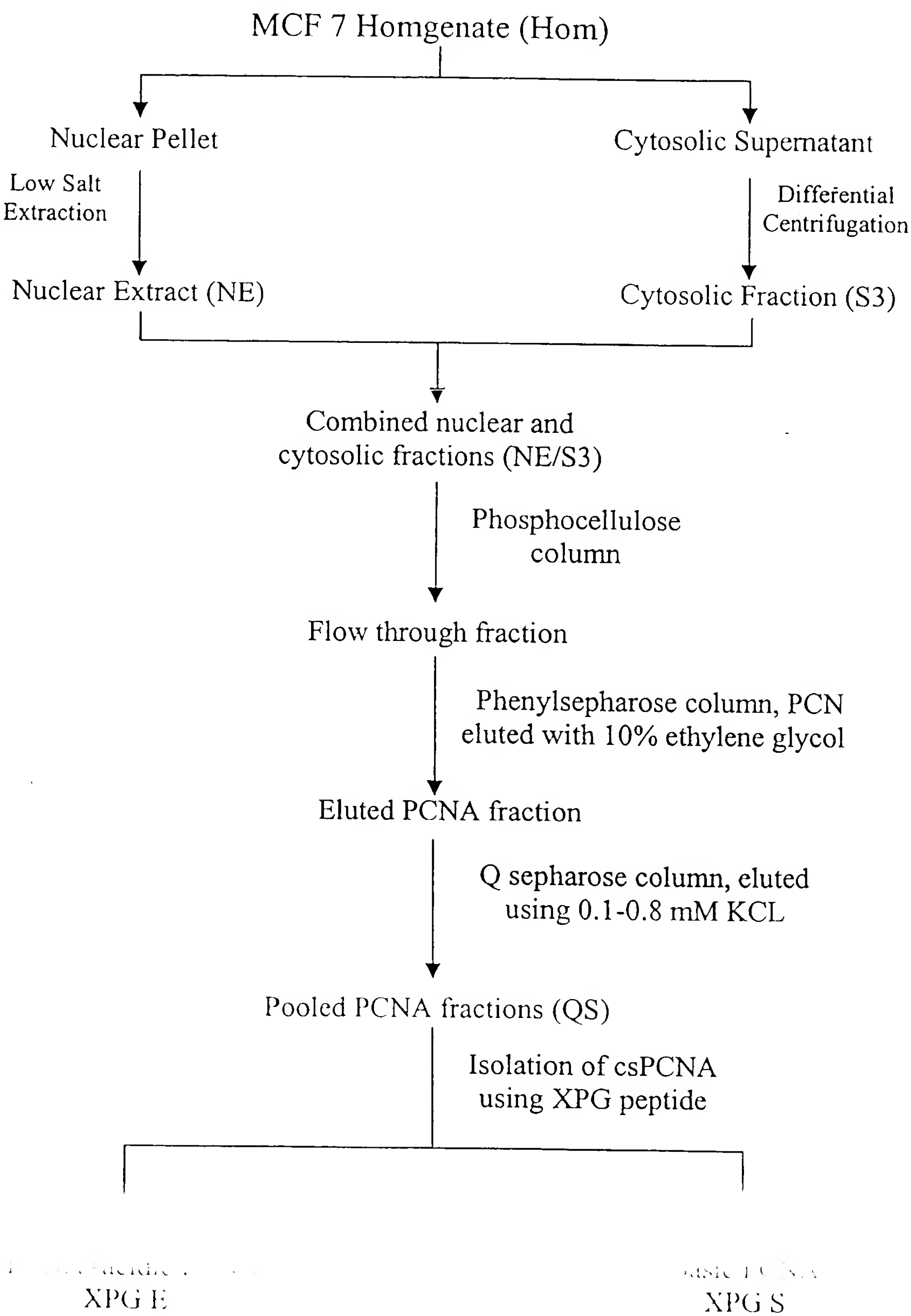

MCF 7 Homgenate (Hom)
Nuclear Pellet
Cytosolic Supernatant
Low Salt Extraction
Differential Centrifugation
Nuclear Extract (NE)
Cytosolic Fraction (S3)
Combined nuclear and cytosolic fractions (NE/S3)
Phosphocellulose column
Flow through fraction
Phenylsepharose column, PCN eluted with 10% ethylene glycol
Eluted PCNA fraction
Q sepharose column, eluted using 0.1-0.8 mM KCL
Pooled PCNA fractions (QS)
Isolation of csPCNA using XPG peptide
XPG E
XPG S

FIGURE 2

:stern Blot Analysis of PCNA Isolation Fractions

FIGURE 3

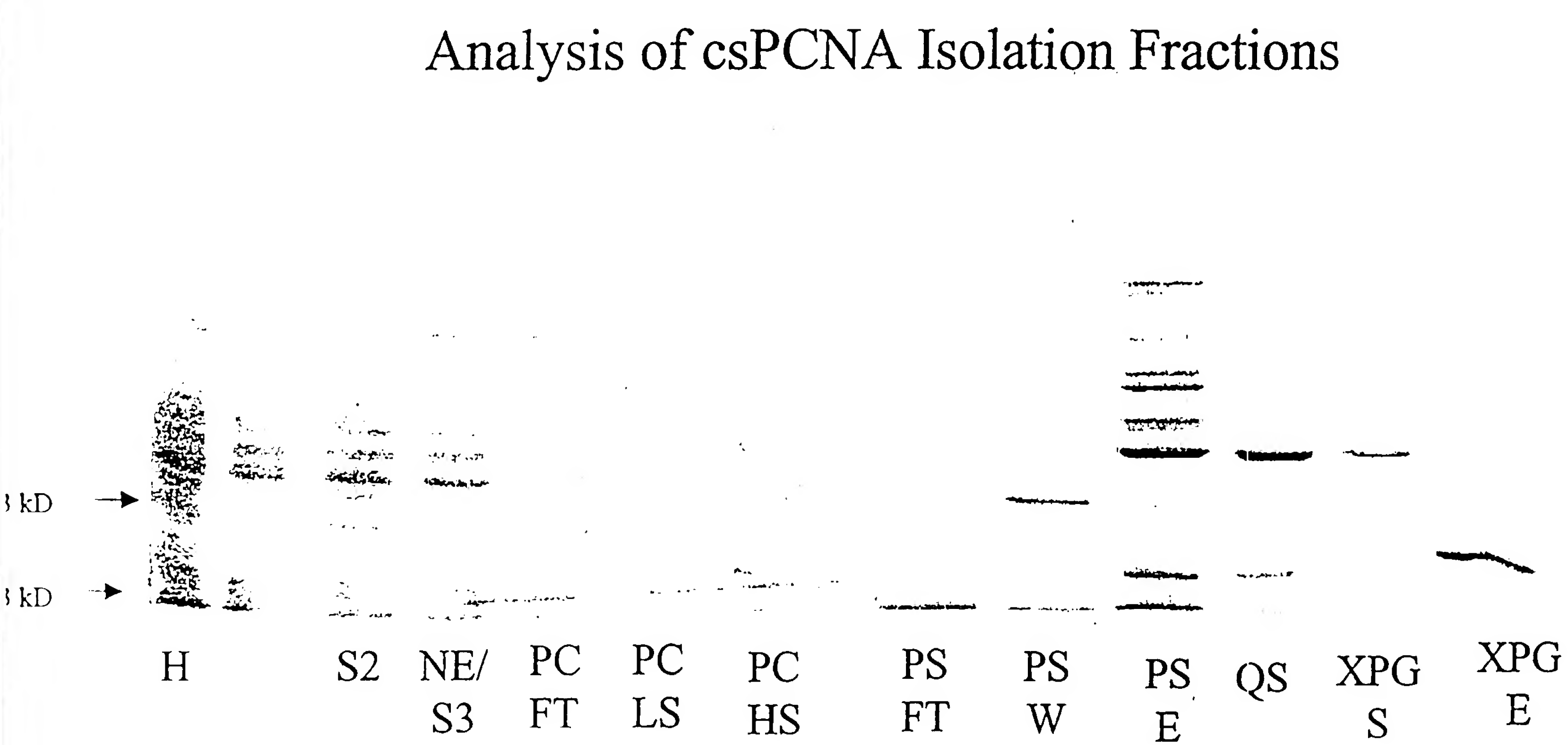

Analysis of csPCNA Isolation Fractions
kD
kD
H
S2
NE/ S3
PC FT
PC LS
PC HS
PS FT
PS W
PS E
QS
XPG S
XPG E

FIGURE 4

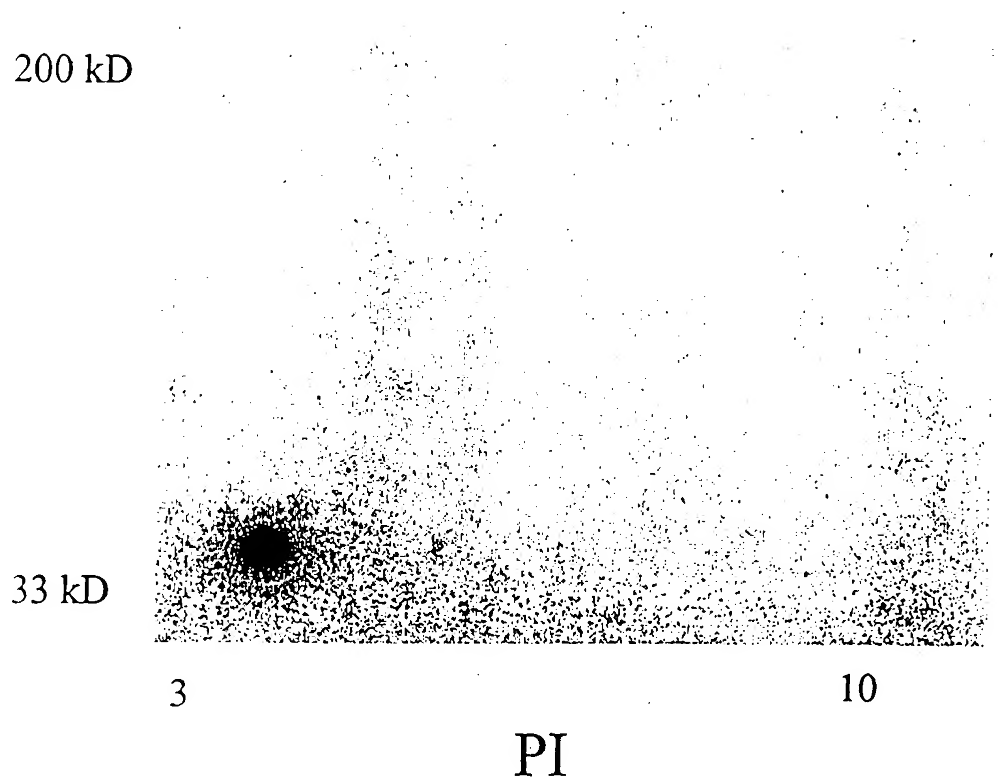
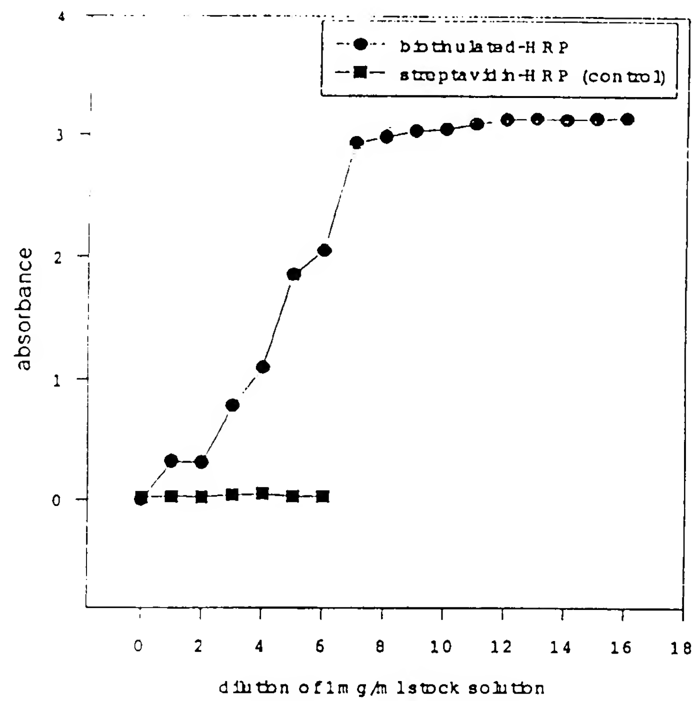


FIGURE 5A

...ion of the viability of the streptavidin surface of the streptavidin-coated plate



0) blank

1) 1:1000 000 dilution

2) 1:800 000 dilution

3) 1: 600 000 dilution

4) 1:400 000 dilution

5) 1:200 000 dilution (2 exsp)

6) 1:150 000 dilution

7) 1:100 000 dilution (2 exsp)

8) 1:75000 dilution

9) 1:50 000 dilution (2 exsp)

10) 1:25 000 dilution (2 exsp)

11) 1:10 000 dilution (2 exsp)

12) 1:5000 dilution

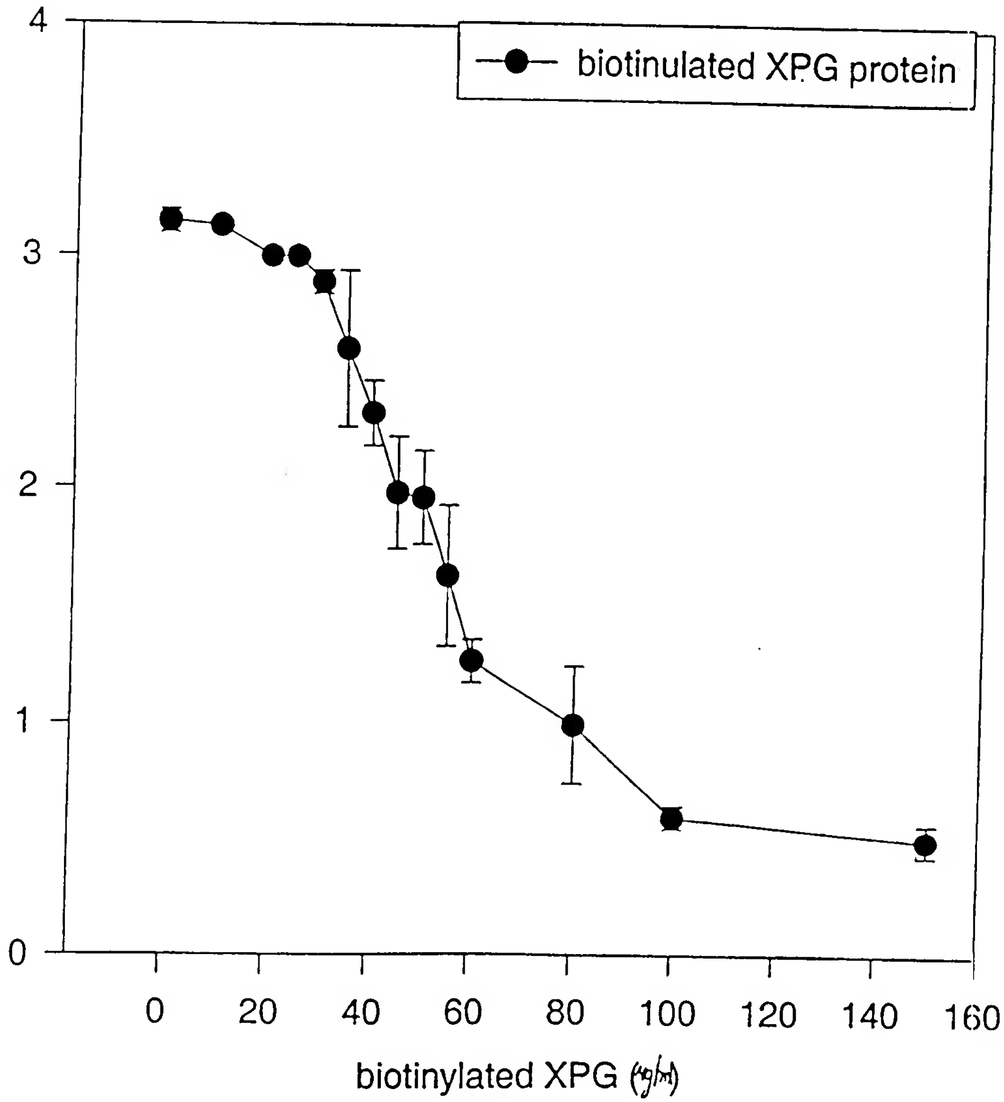
13) 1:1500 dilution

14) 1:1000 dilution

15) 1:100 dilution

16) 1:50 dilution

FIGURE 5B



biotinulated XPG protein
0
1
2
3
4
0
20
40
60
80
100
120
140
160
biotinylated XPG

FIGURE 6

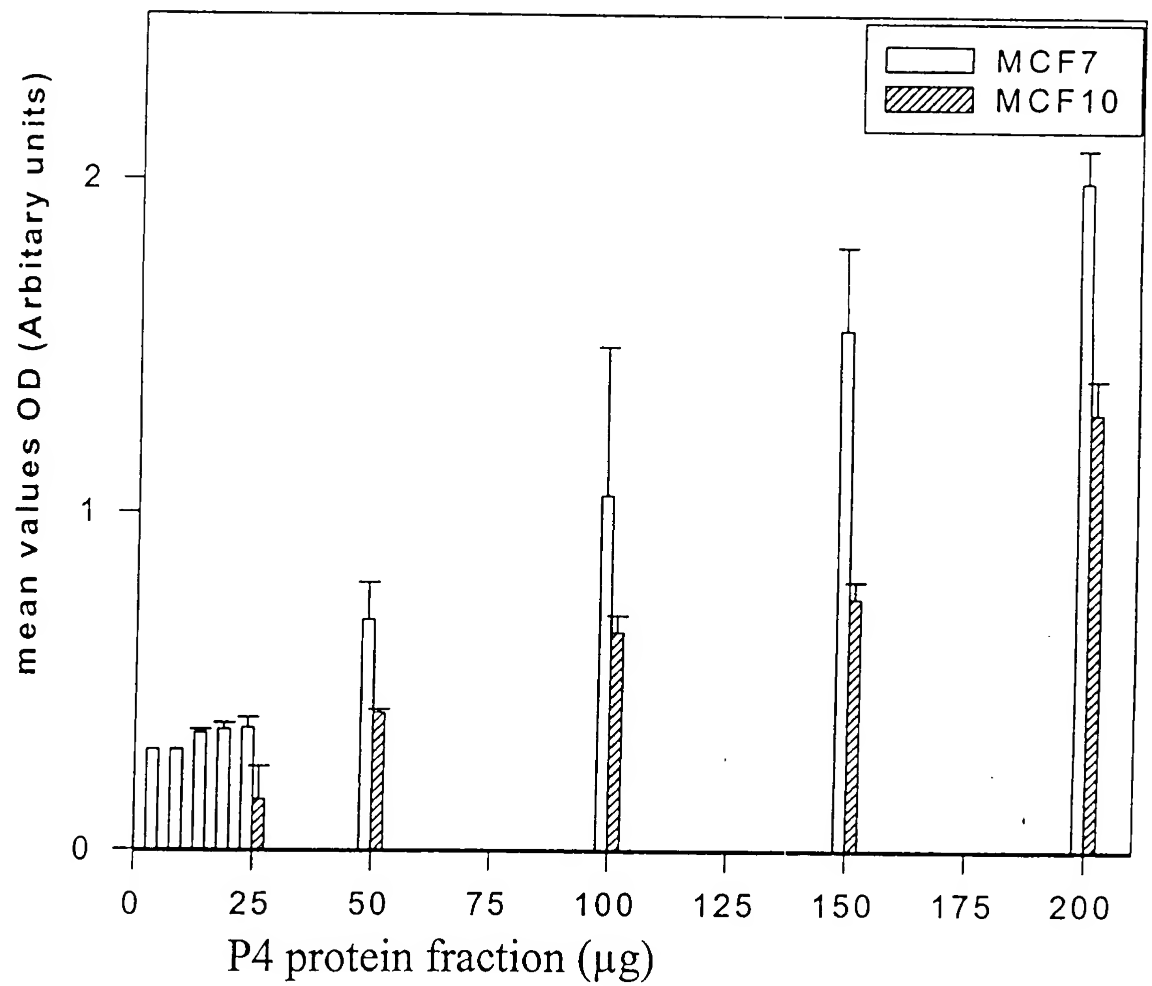

MCF7
MCF10
mean values OD (Arbitary units)
0
1
2
0
25
50
75
100
125
150
175
200
P4 protein fraction (µg)

FIGURE 7A

ELISA ASSAY with MCF7 P4 and MCF10 P4 proteins

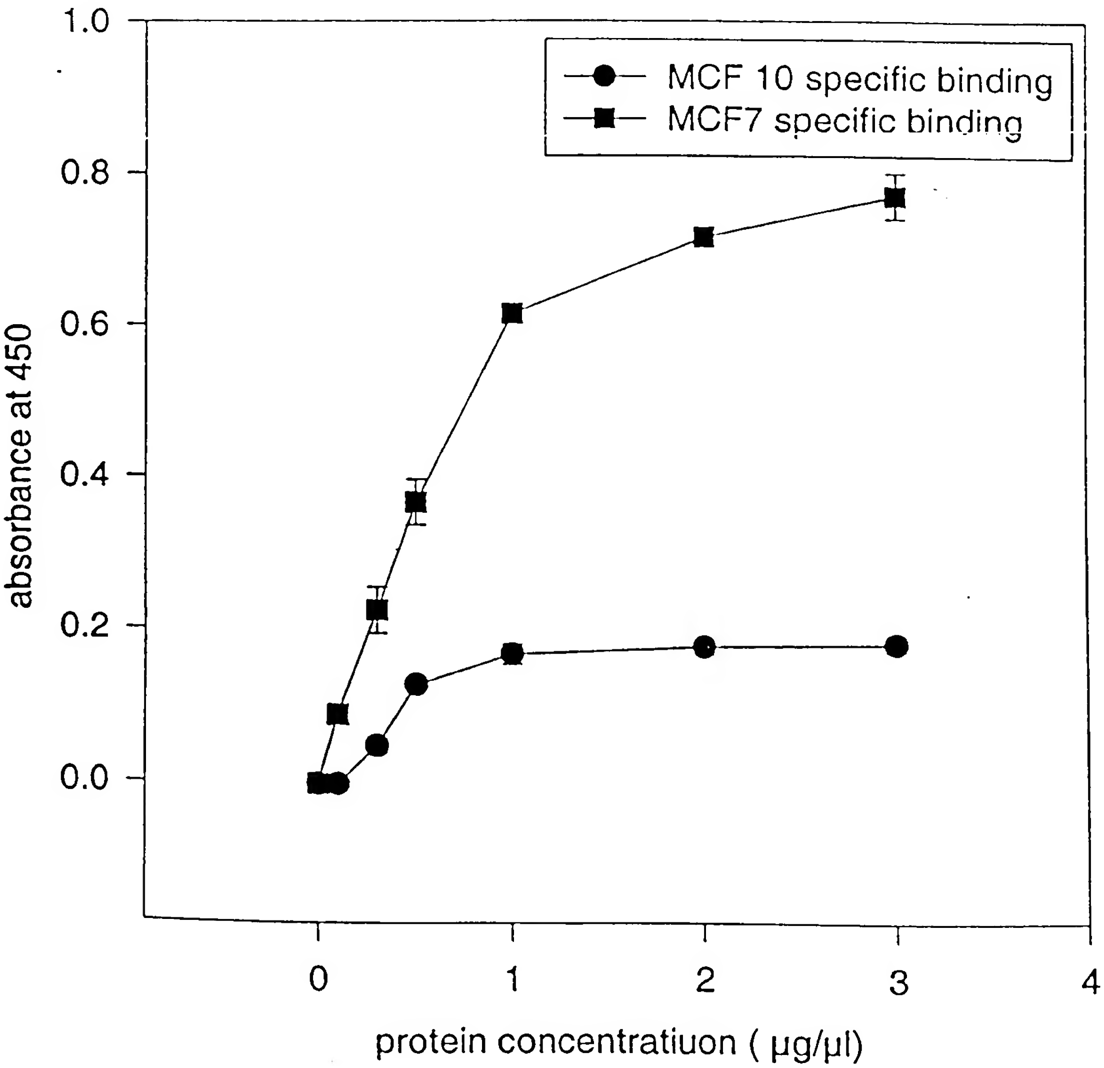
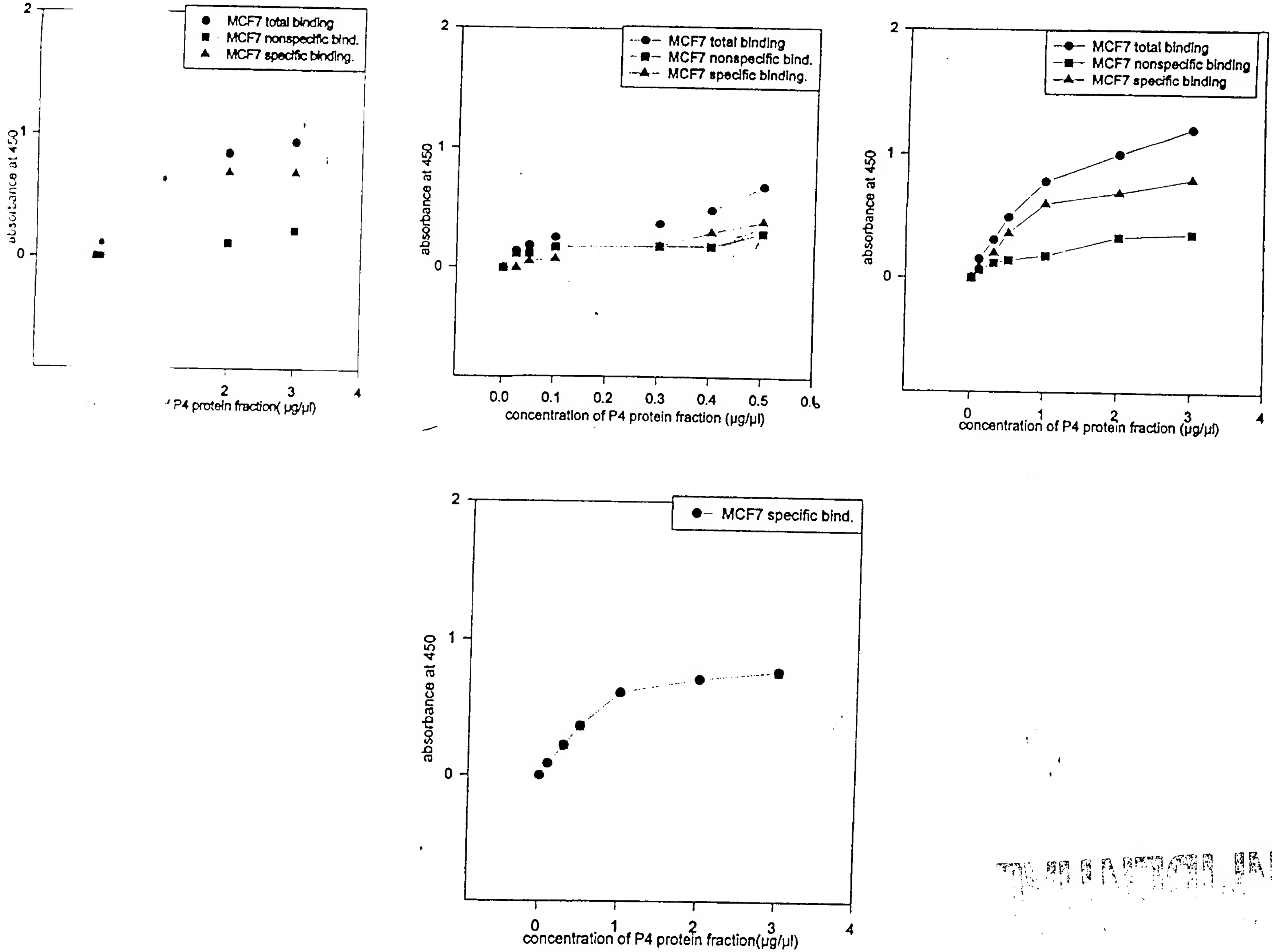
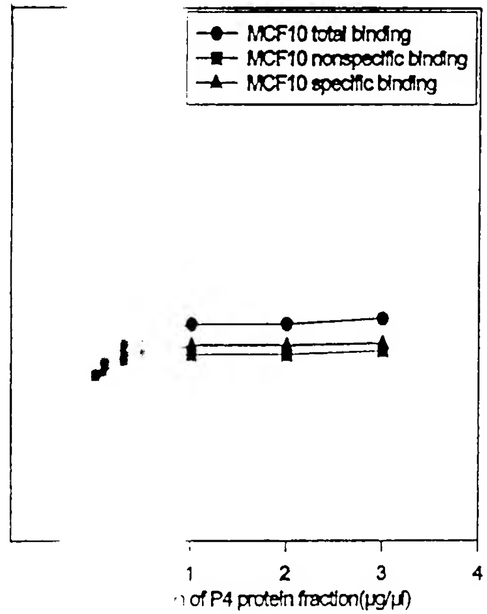


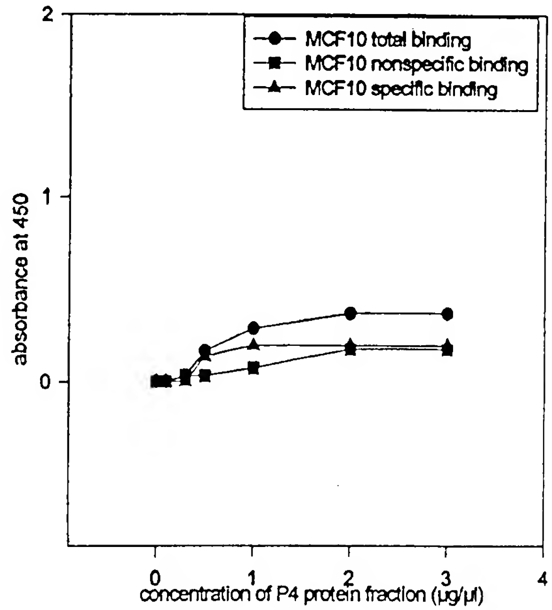
FIGURE 7B

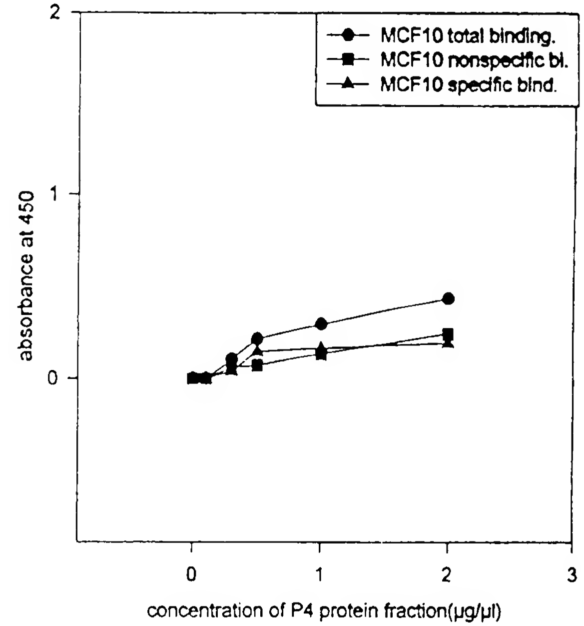


MCF7 total binding
MCF7 nonspecific bind.
MCF7 specific binding.
absorbance at 450
P4 protein fraction(µg/µl)
MCF7 total binding
MCF7 nonspecific bind.
MCF7 specific binding.
absorbance at 450
concentration of P4 protein fraction (µg/µl)
MCF7 total binding
MCF7 nonspecific binding
MCF7 specific binding
absorbance at 450
concentration of P4 protein fraction (µg/µl)
MCF7 specific bind.
absorbance at 450
concentration of P4 protein fraction(µg/µl)

FIGURE 7C


MCF10 total binding
MCF10 nonspecific binding
MCF10 specific binding
1
2
3
4
n of P4 protein fraction(µg/µl)


MCF10 total binding
MCF10 nonspecific binding
MCF10 specific binding
absorbance at 450
2
1
0
0
1
2
3
4
concentration of P4 protein fraction (µg/µl)


MCF10 total binding.
MCF10 nonspecific bi.
MCF10 specific bind.
absorbance at 450
2
1
0
0
1
2
3
concentration of P4 protein fraction(µg/µl)

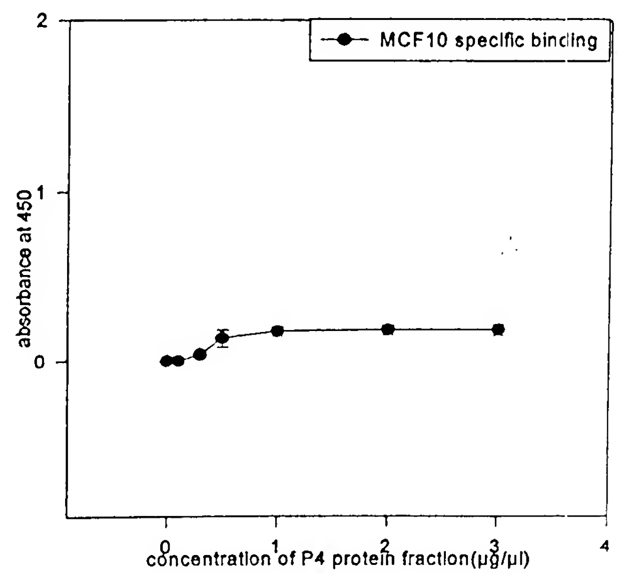
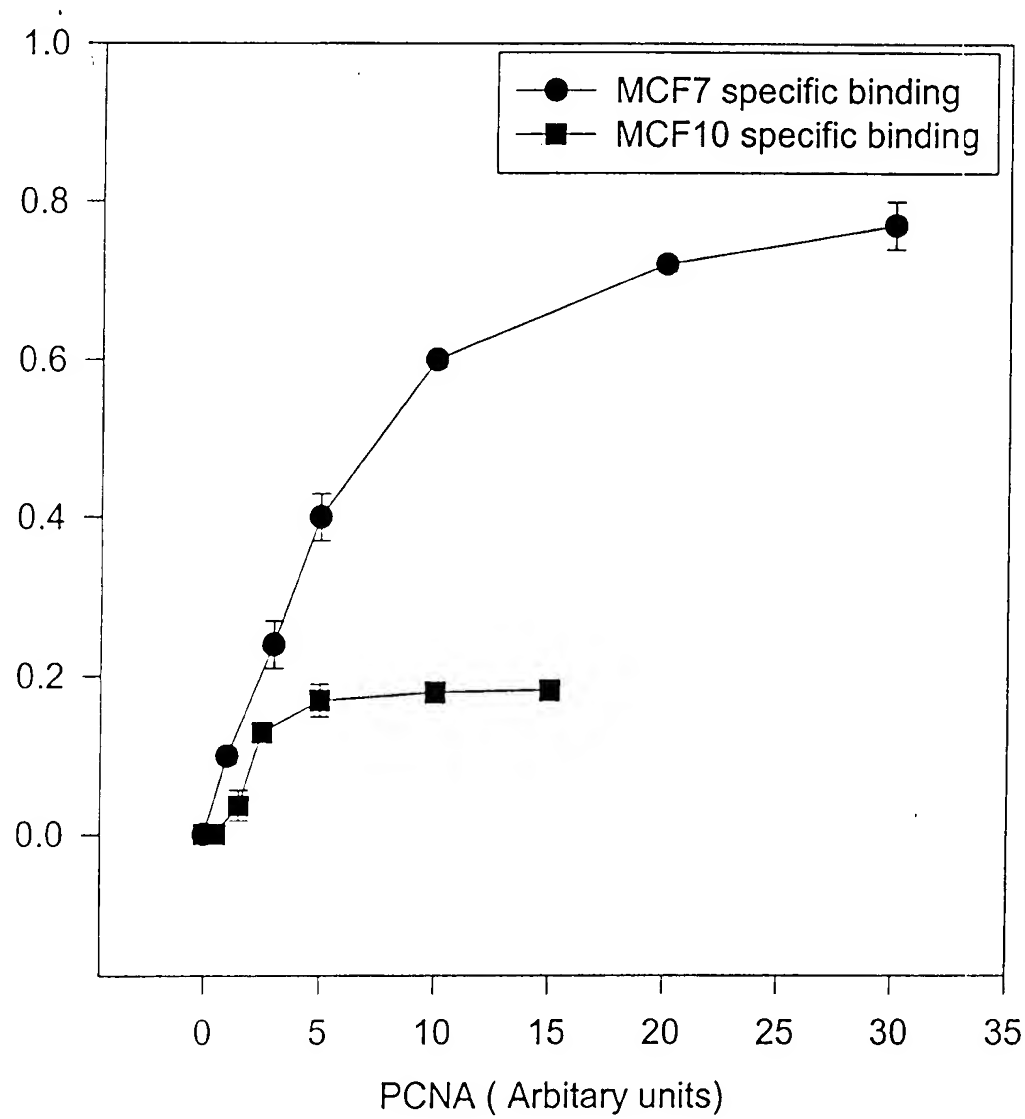

MCF10 specific binding
absorbance at 450
2
1
0
0
1
2
3
4
concentration of P4 protein fraction(µg/µl)

FIGURE 8

ELISA Assay for detection of Cancer Specific Form of PCNA present in MCF breast cancer cell lines



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Delivery attemptTEMPE AZ	01/14/2003 16:18	Customer not available or Business clo
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On FedEx vehicle for deliveryTEMPE AZ	01/14/2003 08:09	
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